**ABSTRACT**

**Introduction:** The aim of this study is to investigate the association between single nucleotide polymorphisms (SNP) in the human promoter of the interleukin-10 (IL-10) gene at positions -1082, -824 and -597 with the development of GDM.

**Methods:** DNA from positions -1082, -824 and -597 in the promoter region of human IL-10 gene was amplified by PCR. Restriction fragment length polymorphism (RFLP) was performed on the purified PCR product to determine genotype and allelic frequencies of the three SNP. Plasma levels of IL-10 in different stages of pregnancy as well as six-weeks postpartum were quantified using enzyme linked immunosorbent assay (ELISA).

**Results:** The results showed that there was a significant (p<0.05) difference in the genotype and allelic frequency of SNP at position -597 between the control and GDM subjects recruited in this study. This was not observed with the other two SNP (-1082 and -824) studied. However, there were no significant differences in plasma IL-10 levels between the two groups.

**Conclusion:** The SNP at position -597 in the promoter of the human IL-10 gene may be associated with development of GDM and it has a potential to be further investigated as a potential tool to be used as a predictive factor using a larger scale trial.

**INTRODUCTION**

Gestational diabetes mellitus (GDM) is defined as carbohydrate intolerance of varying severity with onset or first recognition during pregnancy\[1\]. It is a heterogeneous disorder in which several environmental and genetic factors are implicated\[8\]. The lack of an effective screening tool in predicting GDM presents obstacles in reducing prenatal morbidity and mortality. There is neither worldwide agreement on the cut-off point for diagnosis nor the best way for screening of diabetes in pregnancy\[3\].

Previous reports have shown that maternal insulin resistance plays an important role in the regulation of maternal energy metabolism\[4\]. However, in some women, insulin resistance is more profound and this in turn induces diabetes in pregnancy (GDM). Historically, the mechanisms accounting for the development of peripheral insulin resistance during the course of pregnancy is related to the effects of diabetogenic hormones\[5\]. More recently, investigators have focused on several new potential mediators of insulin resistance including the cytokines\[6,7\]. Interleukin-10 is an immu-
nosuppressive glycoprotein and a major regulatory cytokine of anti-inflammatory responses which can inhibit the production of pro-inflammatory cytokine\cite{8,9}. Recent studies show that a reduction in the capacity to produce IL-10 is associated with some metabolic syndromes and type 2 diabetes mellitus\cite{10,11}. It has been suggested that the genotypic variations in the human IL-10 gene may account for variation in IL-10 production and this in turn may influence susceptibility to diseases \cite{12}. In fact, polymorphisms in the IL-10 gene have been postulated to have a role in determining susceptibility diabetes\cite{13}. However, there is no study studying the association between the polymorphisms in the human IL-10 gene and GDM. Three SNP located with the promoter region of the human IL-10 gene has been widely studied i.e. at positions -597, -824 and -1082. Several reports have linked the presence of these SNP with the control of IL-10 production\cite{14-16}. The aim of current study therefore, is to investigate the association between SNP in the promoter gene of the human IL-10 at positions -597, -824 and -1082 and the development of GDM. The outcome may lead to the development of a reliable predictor of GDM that could be used as a tool to design of better screening methods for early detection of GDM.

**MATERIAL AND METHODS**

Blood was taken from 102 healthy women and 110 GDM women attending the obstetrics clinic of Hospital Tuanku Jaafar (HTJ) (Malaysia) three times during pregnancy (i.e. during second trimester, at 32 and 36 weeks) and once after delivery (six-week post partum) in the course of routine investigations done during pregnancy care.

Both the GDM and normal subjects were recruited after obtaining their consent to participate in the study as well as fulfilling specified inclusion and exclusion criteria. The inclusion criteria for the normal subjects included singleton pregnancy, ages between 18-35 years, parity no more than five, no risk factor for GDM, no glucose intolerance in pregnancy following a 50 g 1-hour glucose challenge screening test (GCT) (glucose < 7.8 mmol/l) at 24-28 weeks of pregnancy and having no history of any medical disorders.

Singleton pregnant women, ages between 18-35 years, parity no more than five, having no history of any medical disorders who exhibited glucose intolerance and/or had risk factors for GDM were subjected to a 75 g glucose tolerance test (OGTT) at 24-28 weeks of pregnancy. All those who were found to have glucose-intolerance according to the WHO criteria\cite{17}, were recruited as subjects for the GDM group. All subjects attended regular follow-up until delivery at the Obstetric Care Centre. In addition, the GDM mothers were appropriately monitored and managed to regulate the blood sugar levels. One hundred and two control women and 110 GDM women completed the study.

The purpose of the study was explained to all the participating women by the attending obstetrician. All women recruited gave informed (signed) consent.

The study protocol was approved by the Research and Ethics Committee of the International Medical University, Malaysia (IMU).

The Plasma and blood cells were separated by centrifugation (1,500 rpm x 10 min 4°C) and stored at -20°C until the analysis. The Genomic DNA was extracted from peripheral blood leukocytes using a genomic isolation DNA commercial kit that was recommended by the manufacturer (QIAGEN, Germany). The extracted DNA was amplified by PCR using published forward and reverse primers\cite{12,13,16} using commercial kits as recommended by the manufacturer (INTRON Biotechnology, Korea). The PCR primers used and the size of the amplified PCR products are shown in TABLE 1. PCR was performed using commercially available PCR premix according to the manufacturer’s recommendation protocol (INTRON Biotechnology, Korea) using a PTC-100 Peltier thermal cycle (MJ Research). Cycling condition for PCR was: 30 cycles at 95°C for 30 seconds, 45°C for 30 seconds, and 72°C for 1 minute. The PCR product was purified using a PCR purification kit as described by the manufacturer (INTRON Biotechnology, Korea).

The purified PCR product was incubated in a thermal bath (DUAL Thermal Bath ALB 128, Korea) at 37°C overnight (EcoNI and RsaI) and at 55°C overnight (MaeIII) (See TABLE 1). For Restriction Fragment Length Polymorphism (RFLP), the PCR digested products were separated by electrophoresis and viewed using a gel documentation system (BioDoc-It Gel documentation System) (UVP, USA). The promoter region of the human IL-10 gene containing the three SNPs, i.e. -1082 G>A, -824 C>T, -597 C>A was successfully genotyped for the all subjects.
The plasma levels of IL-10 were determined at different stages of pregnancy i.e. during the second trimester (24-28 weeks), at 32 and 36 weeks and six-weeks after delivery i.e. post partum for both control and GDM subjects using ELISA according to the manufacturer recommended protocol (eBioscience company, USA).

A statistical analysis using SPSS (version 16) was used for this study. A level of P<0.05 was considered statistically significant. Non-parametric tests were applied for comparison between two study groups. Logistic regression test with an odds ratio and 95% confidence interval (CI) was used to estimate the predictive value.

### RESULTS

The genotype and allele frequencies of the polymorphisms at three positions studied i.e. -597, -824 and -1082 in the promoter region of the human IL-10 gene is shown in TABLE 2. There appeared to be a significant (P=0.03) difference between the GDM and control mothers with respect to genotypic frequency at position -597 of the human IL-10 gene; with an odds ratio of 2.2 (95 % CI 1.21- 4). About 45.5% of the GDM mothers carried the mutant genotype but only 29.4% of the control mothers had this genotype. The frequency of the mutant allele (A-allele) was also significantly (P=0.0001) higher in the GDM mothers when compared to the control mothers with an odds ratio of 2.17 (95 % CI 1.462- 3.216). Therefore, our data seem to suggest that the polymorphism at position -597 of the human IL-10 gene may be associated with the development of GDM in our study population. However, there did not appear to be a significant difference in the genotype and allele frequency of SNP in positions -824 and -1082 between the two study groups.

Haplotype frequencies in both groups were calculated using EH (Estimating Haplotype) program. We found eight different theoretically possible allele combinations in our study groups (See TABLE 3). Our data showed that there was a significant difference (P<0.05) of haplotype frequencies between the control and GDM groups. The CCG haplotype, which consisted of all wild-type allele was detected in higher frequency in the control group. In contrast, the ATA haplotype, which consisted of all mutant-type allele was found to be higher in the GDM subjects. We also detected two rare haplotypes i.e. the ATG and ACA haplotypes.

Plasma IL-10 concentration at different stages of pregnancy (2nd trimester, 32 weeks, 36 weeks and 6-weeks post-partum) was quantified using ELISA. The difference in the plasma IL-10 levels between the both study groups as well as in different stages of pregnancy were compared using Generalized Estimating Equitation (GEE) test. The analysis showed that the differences observed in the plasma levels of IL-10 at the different stages of pregnancy including at post-partum between both the control and GDM subjects were not statistically significant (P>0.05). In both groups, there was a general upward trend in plasma concentration of IL-10 levels from the second trimester of pregnancy up to the third trimester after which the levels appear to drop.

The IL-10 level in each stage of pregnancy was compared with different SNP at three positions in each study group respectively. Although the GDM mothers who carried the homozygous mutant alleles (AA) at position -597 in the promoter region of the human IL-10 gene had apparently lower plasma levels of IL-10 at 32 and 36 weeks of pregnancy compared to the control group, this was to be statistically not significant (P>0.05) (data are not shown).

Logistic regression with an odds ratio and 95% confidence interval were also used to determine the risk and predictive value of SNP in IL-10. Analysis using

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**TABLE 1 : Technical data for the genotyping single nucleotide polymorphism (SNP) in the human IL-10 gene**

<table>
<thead>
<tr>
<th>Position of SNP in IL-10 gene</th>
<th>Restriction Enzyme</th>
<th>Primer Sequence</th>
<th>Allele</th>
<th>Restriction pattern (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1082</td>
<td>EcoNI</td>
<td>Forward: 5’ AAG ACA ACACCTACA AGGCTT CCTT&lt;br&gt;Reverse: 5’ TAA ATA TCC TCA AAG TCCC</td>
<td>Wild-type G</td>
<td>306 + 278</td>
</tr>
<tr>
<td>-824(-819)</td>
<td>MaeIII</td>
<td>Forward: 5’ ATCCAAGAC AAC ACT ACT AA&lt;br&gt;Reverse: 5’ TAA ATA TCC TCA AAG TCC C</td>
<td>Wild-type C</td>
<td>292 + 217 + 79</td>
</tr>
<tr>
<td>-597(-592)</td>
<td>RsaI</td>
<td>Forward: 5’ ATCCAAGACAAC ACT ACTAA&lt;br&gt;Reverse: 5’ TAA ATA TCC TCA AAG TCC C</td>
<td>Wild-type C</td>
<td>306 + 232 + 42</td>
</tr>
</tbody>
</table>

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The aim of this study was to investigate the association between SNP in the human promoter of the IL-10 gene with the development of GDM. Subjects who were carried the mutant-allele at position -597 of the human IL-10 gene are 2.2 times more likely to develop GDM compared to those who carry the wild-type allele at this site. The predictive value was calculated to be only 62% while the sensitivity and specificity of this test was found to be 60% and 57%, respectively.

**TABLE 2: The relationship between genotype and allele frequencies of IL-10 SNP at sites (-597, -824, -1082) in control and GDM groups**

<table>
<thead>
<tr>
<th>Position of SNP</th>
<th>Genotype</th>
<th>Control (n=102) (%)</th>
<th>GDM (n=110) (%)</th>
<th>P value</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>-597</td>
<td>CC</td>
<td>58 (56.9)</td>
<td>44 (40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>14 (13.7)</td>
<td>16 (14.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>30 (29.4)</td>
<td>50 (45.5)</td>
<td>P&lt; 0.05*</td>
<td>2.2 (1.21- 4)</td>
</tr>
<tr>
<td>-824</td>
<td>C allele</td>
<td>130 (63.7)</td>
<td>104 (44.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A allele</td>
<td>74 (36.3)</td>
<td>116 (55.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>19 (18.6)</td>
<td>14 (12.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>46 (45.1)</td>
<td>58 (52.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1082</td>
<td>TT</td>
<td>37 (36.3)</td>
<td>38 (34.5)</td>
<td>P&gt; 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C allele</td>
<td>84 (41.2)</td>
<td>86 (39.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T allele</td>
<td>120 (58.8)</td>
<td>134 (60.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>74 (72.5)</td>
<td>81 (73.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>24 (23.5)</td>
<td>24 (21.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A allele</td>
<td>4 (3.9)</td>
<td>5 (4.5)</td>
<td>P&gt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

*: P value<0.05 was set as statistical significance.

**TABLE 3: Haplotype frequencies of SNP in the promoter of the human IL-10 gene in control and GDM subjects**

<table>
<thead>
<tr>
<th>Haplotype (-597/-824/-1082)</th>
<th>Control (n=102)</th>
<th>GDM (n=110)</th>
<th>( \chi^2 )</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCG</td>
<td>0.2697</td>
<td>0.1784</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCA</td>
<td>0.1085</td>
<td>0.0732</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTG</td>
<td>0.2259</td>
<td>0.1988</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTA</td>
<td>0.0197</td>
<td>0.0184</td>
<td>20.88</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>ACG</td>
<td>0.0361</td>
<td>0.1223</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACA</td>
<td>0.0000</td>
<td>0.0241</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATG</td>
<td>0.3111</td>
<td>0.3498</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATA</td>
<td>0.0288</td>
<td>0.0346</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: P value<0.05 was set as statistical significance.

**DISCUSSION**

The results from this study showed that the frequency of SNP at position -597 in the promoter region of the human IL-10 between control and GDM subjects was significantly different. In addition, the difference between the control and GDM groups in terms of genotype frequency at this site (-597) was also found to be significantly.

Furthermore, the GDM subjects displayed significantly higher frequencies of the mutant–allele at this site (-597) whereas there was a higher frequency of the wild-type-allele in the control subjects. So, our data appears to support the hypothesis that there would be an association between the SNP at position -597 of the human IL-10 gene and the development of GDM. In contrast, we could not demonstrate a similar association with the other two SNP. It was reported that in the patients with type 2 diabetes mellitus, there was a significantly higher frequency of the mutants alleles at po-
position -824 (T-allele) and -597 (A-allele)\cite{13}.

However, in the same patients there were no significant difference in terms of genotype frequency of SNP at these positions between the diabetic mellitus (DM) and control subjects. In another study, it was reported that polymorphisms in the promoter region of the human IL-10 gene at positions -597, -824, and -1082 was associated with insulin resistance and obesity (two risk factors for type 2 diabetes) in non-diabetic subjects but not in type 2 diabetes\cite{19}.

The three SNP (-507, -824, and -1082) in the promoter region of the human IL-10 gene was analysed using “haplotype analysis”. Using this approach, we identified three major haplotypes i.e. CCG, CCA, and ATA in our study population. These haplotypes have also been identified in other populations\cite{20}. The ATA haplotype, which consisted of mutant-type alleles in all three SNPs, was detected in higher frequency in the GDM subjects compared to the control subjects (0.035 vs. 0.029).

Interleukin-10 plays an important role during pregnancy i.e. to prevent any unwanted activation of the mother’s immune system that could be detrimental to the fetus\cite{21-23}. A previous study conducted to assess the physiological effects of pregnancy on the plasma IL-10 concentration, had shown that the plasma levels of IL-10 was significantly higher in healthy pregnant women compared to non-pregnant women\cite{24}. So, it was reassuring that we observed an upward trend in the levels of plasma IL-10 during pregnancy in both groups. The levels increased up to the third trimester, after which this level appear to decrease. This observation is expected as pregnancy is proposed to induce a T-helper-2 (TH2) type of immune response.

However, there was no significant difference in the plasma IL-10 levels between the two study groups in the different stages of pregnancy.

Although the plasma level of IL-10 appeared to be lower in the GDM mothers, it seemed to follow a similar pattern as in control, albeit lower (in GDM subjects). This may be due to the fact that majority of the GDM mothers recruited in this study were controlled with the diet and insulin therapy, where appropriate; therefore they would have similar baseline glucose levels as compared to control subjects. The results may be different if the GDM mothers recruited in this study had uncontrolled or poorly controlled GDM subjects.

However, to date, there are no reports comparing the plasma IL-10 levels between control and GDM mothers. Hence, we are not able to compare our data. In addition, the GDM mothers who carried the homozygous mutant alleles (AA) at position -597 in the promoter region of the human IL-10 gene appeared to have lower levels of plasma IL-10 at 32 and 36 weeks of pregnancy compared to the control group.

However, as the difference was not found to be statistically significant. This observation could be due to a number of factors, including the fact that polymorphisms at position -597 may not be the only factor that can determine production of IL-10. Furthermore, the regulation of IL-10 transcription is currently not well described.

The observation from this study and what has been reported on other biomarkers, it appears that there is no single underlying factor mediating or initiating the onset of GDM. To the best of our knowledge, there has been no study on the association of SNP in the human IL-10 gene and gestational diabetes mellitus, and hence, this study brings new information. Our findings suggest that polymorphism in the human IL-10 gene at position -597 may play a role in determining susceptibility of the GDM. This finding shows promise for use as a predictive factor and needs to be further explored and developed. A large-scale study is warranted.

**ABBREVIATIONS**

ELISA: enzyme linked immunosorbent assay; GDM: Gestational diabetes mellitus; PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism; SNP: Single nucleotide polymorphisms; IL-10: Interleukin 10.

**ACKNOWLEDGMENT**

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**CONFLICT OF INTEREST**

Author does not have conflict of interest with this study.
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